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14. ABSTRACT

Purpose: We are evaluating the efficacy of diclofenac (DFA), an anti-inflammatory agent with L-Selectin sheddase activity, in a murine model of spinal cord injury.

Scope: These studies have focused on the efficacy of DFA in a mouse model of spinal cord injury and in the context of dose, optimal therapeutic window, and dependency on injury severity, using clinically relevant outcome measures that include neurologic assessments and assays of bladder function.

Major findings:

- 40 mg/kg, i.p. of DFA is the minimally effective dose to induce L-selectin shedding after spinal cord injury.
- 40mg/kg DFA, i.p, administered up to at least 3 hours post injury results in a significant improvement in locomotor recovery after either mild or severe injuries, a finding that speaks to the robust therapeutic effect of this drug.
-There were no adverse effects to animal health, as evaluated by body weight.
-There was no added locomotor recovery due to multiple, successive doses of DFA. Moreover, additional doses proved to be toxic and increased animal mortality
-40 mg/kg, i.p. of DFA, given up to 3 hours post injury, resulted in a long-term improvement in white matter sparing and reduced lesion volume.
-While DFA did not improve long-term bladder function, nor did it exacerbate bladder dysfunction after injury.

Significance: We have identified robust locomotor recovery in both mild and severe spinal cord injured mice that received DFA up to 3 hours following injury. Furthermore, we identified no adverse effects utilizing this dose. Therefore, these promising data suggest that DFA, administered within at least 3 hours of spinal cord injury, could be an effective therapeutic intervention for human spinal cord injury.

We recognize that the dose in mice far exceeds what is safe for humans. However, others have studied L-selectin shedding induced by DFA that was administered in humans.[1] A dose of 50 mg/individual, well within the safety profile for humans, resulted in a similar level of L-selectin shedding as what we reported for mice at the much higher dose of 40mg/kg. Thus, while this likely speaks to species differences in dosing, the principals we have posited still remain translatable to humans. DFA continues to be a promising therapeutic for those who have sustained an acute spinal cord injury.

[1]In vivo effect on diclofenac potassium L-selectin expression by polymorphonuclear leukocytes. By: Baranda, Lourdes; Abud-Mendoza, Carlos; Portales-Perez, Diana Patricia; Layseca, Esther; De La Fuente, Hortensia; Amaro, Roberto Gonzalez; Ibarra,Jose. Mexican Journal of Rheumatology. May jun1998, Vol. 13 Issue 3, p144. 6p. 3 Charts, 2 Graphs. Language: Spanish. (AN:3748173)

15. SUBJECT TERMS

spinal cord injury, L-Selectin, diclofenac, mouse, urologic function, neurologic function

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INTRODUCTION

This proposal is investigating the hypothesis that the anti-inflammatory drug diclofenac (DFA), acting as an L-selectin sheddase, will improve neurologic outcome and ameliorate neurogenic bladder dysfunction resulting from spinal cord injury (SCI). L-selectin is expressed on the surface of all leukocytes. Preliminary data using the L-selectin knockout (KO) mouse confirmed the dependency of L-selectin on neurologic recovery and thus served as the basis for pharmacologic targeting of this molecule in a murine model of SCI. The specific aims of this proposal are to define the minimal effective dose of DFA, the optimal window of therapeutic intervention for DFA, whether DFA administration improves bladder function, and if the efficacy of DFA is dependent on proteolytic cleavage of L-selectin.

Please note that each task is indicated in bold.

KEY WORDS
spinal cord injury, L-selectin, inflammation, diclofenac, shedding, mouse, urologic function, neurologic function

ACCOMPLISHMENTS

Specific Aim 1

Task 1. Define the minimal effective dose of DFA

1a. Obtain animal use protocol approval (months 1-4)

We received approval from the UCSF IACUC and ACURO to conduct these studies.

1b. Assay L-Selectin sheddase activity in both blood and spinal cord by flow cytometry at 8 hours to 7 days after a single bolus administration of DFA (at 1, 5, 10, 20, or 40 mg/kg) given immediately after injury (months 5-9)

To assess the L-selectin sheddase activity of increasing doses of DFA, male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (moderate injury). DFA (60mg/kg, 40mg/kg, 20mg/kg, 10mg/kg, 5mg/kg, or 1mg/kg) or vehicle (PBS) was administered immediately following spinal cord injury (SCI). L-selectin sheddase activity was quantified by utilizing an enzyme-linked immunosorbent assay (ELISA) that measures the amount of soluble L-selectin in plasma and spinal cord tissue (n=5/group) collected at 8 hours, 1 day, 3 days, and 7 days following SCI/DFA administration. Data from plasma samples are summarized in Figure 1A-D and data from spinal cord tissue are summarized in Figure 1E-H. Significance for all data was determined using a one-way ANOVA followed by a Dunnett's post-hoc test and was defined as $p<0.05$.

We identified a significant increase in the levels of soluble L-selectin in the plasma of mice receiving 60mg/kg and 40mg/kg DFA at 8 hours (Figure 1A) and 1 day (Figure 1B) post-injury/administration. By 3 days (Figure 1C), this difference had returned to baseline and continued to be non-significant at 7 days (Figure 1D). The increased levels of soluble L-selectin are indicative of increased L-selectin sheddase activity and suggest that the 60mg/kg and 40mg/kg DFA doses were potent within the first 24 hours of administration. No other doses showed a significant alteration to the soluble levels of L-selectin, suggesting a lack of potency.
We also identified a significant increase in the levels of soluble L-selectin in the spinal cords of mice receiving 60mg/kg and 40mg/kg DFA at 8 hours (Figure 1E) and 1 day (Figure 1F) post-injury/administration. As with the plasma data, this difference had returned to baseline at 3 days (Figure 1G) and was still non-significant at 7 days (Figure 1H). These data corroborate the data from the plasma and furthermore indicate that the L-selectin sheddase activity of 60mg/kg and 40mg/kg DFA is occurring at the target site of interest: the injured spinal cord. As with the plasma data, lower doses of DFA exhibited no significant changes to L-selectin levels in the injured spinal cord. **Taken together, these data suggest that the 40mg/kg dose of DFA is the minimum effective dose required for L-selectin sheddase activity in mice.**

We next confirmed the shedding activity of 40mg/kg DFA by performing flow cytometry to detect the loss of L-selectin on leukocytes 24 hours post-SCI. In preliminary studies, male C57BL/6 mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (mild injury). DFA (40 mg/kg) or vehicle was administered immediately following spinal cord injury (SCI) (n=5/group). Plasma and spinal cord tissue was collected at 24 hours post-SCI/DFA administration and processed by flow cytometry using markers to broadly identify leukocyte populations. Data from plasmid samples processed by flow cytometry are in Figure 2A, and data from spinal cord samples processed by flow cytometry are in Figure 2B. Significance was defined as $p<0.05$ and determined using a one-way ANOVA followed by a Dunnett's post-hoc test for flow data. Using flow cytometry, we identified a significant reduction in L-selectin on circulating leukocytes from plasma at 1 day post-SCI/DFA administration (Figure 2A) and on leukocytes from the spinal cord at 1 day post-SCI/DFA administration (Figure 2B).

To validate the preliminary findings in a severe model of SCI, we repeated the above studies in male C57BL/6 mice that were subjected to a 3g weight dropped 5 cm onto the exposed spinal cord at the thoracic 9 vertebral level (severe injury). DFA (40 mg/kg) or vehicle was administered immediately following SCI (n=7/group). Plasma and spinal cord tissue was collected at 24 hours post-SCI/DFA administration and processed by flow cytometry using more specific markers for myeloid lineage cells and subtypes including, neutrophils and monocytes. Plasma from uninjured control mice was also analyzed to establish baseline L-selectin values prior to SCI (n=4/group). We identified reduced L-selectin specifically on neutrophil and non-classical monocyte subtypes in the plasma and in the injured spinal cord (Figure 3A-B). **Taken together, these data suggest that 1) L-selectin sheddase activity of DFA in the spinal cord can be monitored by utilizing either flow cytometry to observe reductions in L-selectin expression in leukocytes or ELISA to observe increases in soluble L-selectin that has been shed by circulating leukocytes. 2) 40mg/kg DFA is the minimally effective dose to selectively induce L-selectin shedding from leukocyte subtypes following SCI in mice.**

1c. Use a similar dosing strategy and compare neurologic recovery in spinal cord injured mice treated with DFA or vehicle immediately after SCI (months 10-12).

Male C57BL/6 mice were subjected to a mild injury (2g weight dropped 7.5 cm onto the exposed spinal cord) at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered immediately following SCI (n=15/group), using a randomized, blinded design. Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion weekly 6 weeks post-injury (Figure 4A). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$), but no effect for treatment ($p>0.05$) based upon the BMS. We also examined several additional tests that may detect more subtle changes after mild SCIs. We classifed mice based on the ability to frequently/consistently plantar step as a method of assessing this portion of the BMS scale (Figure 4B). Frequent/consistent plantar stepping was observed in 40% of vehicle mice vs. 92% of 40mg/kg DFA mice. Chi square frequency analysis with fisher's exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA ($p<0.05$). **Thus, mice receiving 40mg/kg DFA had improved plantar stepping consistency.** To further investigate locomotor recovery, at 6 weeks post-injury, mice were tested for coordination by walking across a grid for 3 minutes. One mouse from the vehicle group was excluded due to the inability to step. The number of foot faults, indicative of a lack of coordination, was normalized to the total distance travelled by the mouse (Figure 4C). A Kruskal-Wallis test demonstrated a significant reduction in errors made by mice receiving 40mg/kg DFA ($p<0.05$). **These data demonstrated that DFA improved overall coordination of mice following mild SCI.**

Together, these data demonstrate that mice with mild SCI and receiving 40mg/kg DFA showed significant neurological recovery relative to the vehicle controls.

Mice were weighed prior to injury, 1 and 3 days post-injury, and then weekly thereafter (Figure 5A). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$), but no effect for treatment ($p>0.05$). Notably, mice lost weight shortly after SCI, then regained this weight, ultimately ending with a higher weight than prior to surgery due to aging. **These data demonstrate that DFA administration did not adversely affect animal weight and overall health in a mild-SCI paradigm.**

Next, we sought to evaluate bladder function in DFA-treated mice by awake cystometry in a subset of mice (n=9/group). Consistent with this being a mild injury paradigm, bladder functional recovery may have

reached a ceiling effect, similar to the observations of locomotor recovery. When cystometry was performed at the end of the study, 29/34 (~85%) of mice exhibited partial or complete voluntary bladder voiding during daily animal care. Following euthanasia, bladders were removed, weighed, and normalized to total bodyweight (Figure 5B). A one-tailed t-test demonstrated no significant differences on normalized bladder weights between groups ($p>0.05$). Following SCI, bladder dysyngergia typically results in alterations to the levels of smooth/skeletal muscle, as well as connective tissue matrices, leading to a thickening of the bladder wall. However, all SCI mice in this study, regardless of treatment, demonstrated normalized bladder weights comparable to historical uninjured controls. Therefore, any potential DFA-dependent effects on bladder recovery may have been obscured by this degree of baseline recovery. The volume of residual urine (Figure 5C) and the number of uninhibited bladder contractions/voiding cycle (Figure 5D) for all mice have been calculated, and the remaining analysis of cystometry data is currently in progress. A Kruskal-Wallis test for each measure demonstrated no significant differences for the volume of residual urine or number of uninhibited bladder contractions/voiding cycle between groups ($p>0.05$). **Taken together, these data suggest that urologic function is not being altered by DFA in this mild injury paradigm. However, the ability to detect DFA-dependent effects on urological function could be increased in a more severe SCI paradigm that has a wider range of recovery.**

1d. Conduct morphometric analyses (spared white matter, glial scarring, serotonergic fiber tracts) of the cords, prepared from animals in 1c (months 13-15).

Due to the challenges in assessing locomotor recovery with a mild SCI, we elected to analyze tissue from mice receiving a more severe SCI from Task 2c. Male C57BL/6 mice were subjected to a 3g weight dropped 5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg; n=14) or vehicle (n=7), was administered 3 hours following SCI using a randomized, blinded design. At 6-weeks post-injury spinal cords were harvested and sectioned for histological analysis. Cross-sections from one cohort were eriochrome stained, imaged, and analyzed for white matter sparing and lesion volume (Figure 6). A Mann-Whitney test demonstrated that **mice with severe SCI that received 40 mg/kg DFA treatment at 3 hours after injury exhibited increased the total white matter volume ($p<0.05$) with reduced total lesion volume compared to vehicle-treated animals ($p<0.05$)**. **At the injury epicenter, white matter volume was significantly greater in DFA-treated animals compared to animals receiving a vehicle injection ($p<0.01$). No significant difference was observed in the lesion volume at the epicenter ($p=0.1903$).**

To assess serotonergic fiber tracts**,** spinal cords from a second cohort were sectioned in the longitudinal plane, stained for serotonergic fiber tracts (5-HT), and analyzed for estimated fiber length (Figure 7, n=13-14/group). A Mann-Whitney test demonstrated that **mice with severe SCI that received 40 mg/kg DFA treatment at 3 hours after injury exhibited a strong trend towards greater serotonergic fiber length at 6 weeks post-SCI ($p=0.054$).**

Specific Aim 2

Task 2. Determine the optimal window of therapeutic intervention for DFA.
2c. Determine if optimal dosing of DFA, defined in 2b, supports neurologic recovery after a more severe SCI (months 24-26).

Due to the challenges in assessing locomotor recovery with a mild SCI (discussed above), we elected to move to a more severe SCI model prior to performing tasks 2a and 2b. Critically, mice receiving a severe SCI lose the ability to perform weight supported steps, providing an ideal model to assess locomotor recovery. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered immediately following SCI (n=15/group), using a randomized, blinded design.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 8A). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$) and for treatment ($p<0.0001$). A Sidak's multiple comparisons test demonstrated that **mice with severe SCI that received 40mg/kg DFA immediately after**

injury exhibited improved locomotor recovery starting at 3 days post-SCI ($p<0.05$) and persisting until 6 weeks ($p<0.0001$).

We next sought to classify mice based on the ability to step as a method of assessing recovery (Figure 8B). Stepping was observed in 8% of vehicle mice vs. 69% of 40mg/kg DFA mice. Chi square frequency analysis with fisher's exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA ($p<0.01$). **Thus, mice receiving 40mg/kg DFA had improved ability at stepping.**

Mice were weighed prior to injury, 1 and 3 days post-injury, and then weekly thereafter (Figure 8C). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$), but no effect for treatment ($p>0.05$). Notably, mice lost weight shortly after SCI, and ultimately never regained full weight. This is likely due to the severity of the injury affecting muscle tone. Following euthanasia, bladders were removed, weighed, and normalized to total body weight (Figure 8D). A one-tailed t-test demonstrated no significant differences on normalized bladder weights between groups ($p>0.05$). **These data demonstrate that DFA administration did not adversely affect animal weight and overall health in a severe SCI paradigm. Furthermore, bladder thickness and weight did not appear to be influenced by DFA.**

Taken together, these data demonstrate that administration of 40mg/kg DFA immediately after a severe SCI supports locomotor recovery.

2a. Evaluate long-term neurologic recovery in mice after a moderate level of SCI that have been treated with a bolus injection of DFA or vehicle at 3 hours. If there is benefit, then repeat study but initiate treatment at 8 hours. If there is no benefit at 3 hours, then repeat study with DFA/vehicle given at 1 hour (months 16-20).

Given the success with the more severe model of SCI in 2c (discussed above), we elected to continue using this model for the remaining aims as it represents a better model at differentiating locomotor recovery and is more clinically relevant. We next evaluated the efficacy of DFA administration 3 hours following a severe SCI. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered 3 hours following SCI (n=15/group), using a randomized, blinded design.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 9A). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$) and for treatment ($p<0.001$). A Sidak's multiple comparisons test demonstrated that **mice with severe SCI that received 40mg/kg DFA immediately after injury exhibited improved locomotor recovery starting at 1 week post-SCI ($p<0.05$) and persisting until 6 weeks ($p<0.001$).**

We next sought to classify mice based on the ability to step as a method of assessing recovery (Figure 9B). Stepping was observed in 18% of vehicle mice vs. 62% of 40mg/kg DFA mice. Chi square frequency analysis with fisher's exact test demonstrated a significant effect between mice receiving vehicle and 40mg/kg DFA ($p<0.05$). **Thus, mice receiving 40mg/kg DFA had improved ability at stepping.**

Taken together, these data demonstrated that administration of 40mg/kg DFA 3 hours after a severe SCI supports locomotor recovery.

Accordingly, we next evaluated locomotor recovery in mice that received DFA 8 hours following a severe SCI. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered 8 hours following SCI (n=15/group), using a randomized, blinded design.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 10A). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$) and no effect for treatment ($p>0.05$), demonstrating that **mice that received DFA 8 hours post-severe SCI did not exhibit locomotor recovery.**

We next sought to classify mice based on the ability to step as a method of assessing recovery (Figure 10B). Stepping was observed in 15% of vehicle mice vs. 36% of 40mg/kg DFA mice. Chi square frequency analysis with fisher's exact test demonstrated a no significant effect between mice receiving vehicle and

40mg/kg DFA ($p>0.05$). **Thus, mice receiving 40mg/kg DFA 8 hours post-SCI did not exhibit improved ability at stepping.**

These collective data demonstrate that administration of 40mg/kg DFA 8 hours after a severe SCI does not support locomotor recovery. Therefore, the optimal therapeutic window for efficacy is within a time frame of less than 8 hours post injury. Thus, while efficacy is clearly evident when treatment is initiated at 3 hours of injury, our findings do not rule out the possibility that DFA given after 3 hours but before 8 hours might likewise prove beneficial. Such a distinction becomes important given the challenges in the field where accessibility to treatment may be delayed.

2b. Assess neurologic recovery using multiple doses of DFA, as defined in 2a. Compare mice that are given DFA 1 day after the first dose, 1 and 2 days after the first dose, and 1,2, and 3 days after the first dose (months 21-22).

We next sought to define whether multiple doses of 40mg/kg DFA, administered within the therapeutic window at 3 hours and successively for up to 3 days. Male C57BL/6 mice were subjected to a 3g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg) or vehicle was administered 3 hours following SCI, and then a subset of DFA-treated mice received either one 40mg/kg DFA dose 24 hours post-SCI or two 40mg/kg DFA doses at 24 and 48 hours post-SCI(n=15/group), using a randomized, blinded design.

As we performed the study, we observed high mortality in certain animals. Experimenters, not blinded to groups, determined that mortality occurred primarily in mice receiving multiple doses of DFA (Figure 11A). 100% of mice that received 3 total doses of DFA (3 hours, 1 day, and 3 days post-SCI) died. 47% of mice that received 2 total doses of DFA (3 hours and 1 day post-SCI) died. However, vehicle and single dose DFA-treated mice at 3 hours did not exhibit increased mortality outside normal levels (~10-15%). **These data suggest multiple doses of 40mg/kg DFA are toxic.** We elected to continue assessing behavior in the remaining mice as a replication of the previous data and to observe if the surviving subset of mice (n=7) exhibited locomotor recovery.

Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion 3 hours, 1 day, 3 days, and then weekly for 6 weeks post-injury (Figure 11B). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$) and for treatment ($p<0.01$). A Sidak's multiple comparisons test demonstrated that **mice with severe SCI that all 40mg/kg DFA treated groups (single and double doses) exhibited locomotor recovery starting at 1 week post-SCI ($p<0.001$ for single and $p<0.01$ for double) and persisting until 6 weeks ($p<0.0001$ for single and $p<0.05$ for double). Critically, there were no significant differences ($p>0.05$) between single and double-dose DFA groups, indicating no conferred benefit to additional doses.**

Taken together, these data demonstrated that administration of multiple doses of 40mg/kg DFA after a severe SCI do not confer any added benefits over a single dose of 40mg/kg DFA. Moreover, multiple doses increase the risk of toxicity and adverse side-effects, indicating a single dose regimen of 40mg/kg DFA is most appropriate clinically.

Specific Aim 3

Task 3. Determine if DFA improves bladder function

3a. Using optimal dosing defined in 2c, compare urologic function in spinal cord injured mice treated with either vehicle or DFA. (months 27-29).

We have previously shown that long-term neurological recovery is improved in our optimal dosing regimen: a severe model of SCI with the minimally defined dose of DFA (40 mg/kg), administered 3 hours following injury (Task 2c). To determine of DFA treatment improves urologic function in the sever model of SCI, male C57BL/6 mice were subjected to a 3g weight dropped 5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. DFA (40mg/kg; n=15) or vehicle (n=13) was administered 3 hours following SCI using a randomized, blinded design. At 7-weeks post-injury awake cystometry was performed (Figure 12). An unpaired T-test demonstrated that **mice with severe SCI that received 50 mg/kg DFA treatment at 3 hours after**

injury exhibited no significant improvements compared to vehicle-treated control mice in several measures of bladder function including intermicturition interval (p=0.2), threshold pressure (p=0.4), minimal and maximum voiding pressure (p=0.3 and 0.6, respectively), residual urine (p=0.8), voiding efficiency (p=0.1), and the occurrence and pressure amplitude of non-voiding contractions (p=0.1 and 0.4 respectively).

Taken together, these data demonstrate that mice with severe SCI receiving 40mg/kg DFA at 3 hours post-injury do not show significant improvement in urologic function compared to the vehicle controls.

Specific Aim 4

Task 4. Determine if efficacy of DFA is dependent on its proteolytic cleavage of L-selectin.

To assess the dependence of DFA-induced cleavage of L-selectin on the functional recovery observed in Tasks 1 and 2, we obtained L(E) same mice from a collaborator at another institution. The mice were successfully rederived and breeding pairs for set up. Endogenous shedding of L-selectin has been previously shown in response to painful stimuli and may play a role in inflammation following SCI. Therefore, an altered response to SCI may occur in L(E) same mice that are resistant to L-selectin shedding. Prior to assessing the efficacy of DFA in L(E) same mice, we compared neurological recovery following moderate-severe SCI in L(E) same versus WT mice. Male C57BL/6 (n=10) and L(E) same (n=7) mice were subjected to a 2g weight dropped 7.5 cm onto the exposed spinal cord at the thoracic 9 vertebral level. Neurologic recovery was measured using the Basso mouse scale (BMS), where 0 indicates complete hind-limb paralysis and 9 indicates normal locomotion. Testing was performed in a blinded fashion weekly 6 weeks post-injury (Figure 13). A two-way repeated measures ANOVA demonstrated a significant effect for time ($p<0.0001$), but no effect for genotype ($p>0.05$). A Sidak's multiple comparisons test demonstrated **no significant difference between L(E) same mice versus wild-type mice with moderate-severe SCI at all time points ($p>0.05$).**

Given the negative findings, we examined the resistance of L(E) same mice to L-selectin shedding by treated uninjured mice with DFA. Plasma was collected at 8 hours after DFA administration and processed by flow cytometry (Figure 14A). Significance was defined as $p<0.05$ and determined using a two-tailed Student's T-test. We identified **a modest, but significant reduction of 14.1% in L-selectin on circulating neutrophils in L(E) same mice treated with 40 mg/kg DFA.** These results prompted us to investigate the parent transgenic mouse lines, L(E) neo and L(E) homo, that are crossed to generate the L(E) same genotype. Leukocytes in L(E) neo mice have been previously shown to express irregular levels of L-selectin, therefore, we focused solely on L(E) homo mice. Similar to the previous experiment, DFA was administered to uninjured L(E) homo mice and plasma was collected at 8 hours after DFA administration for flow cytometry analysis (Figure 14B). **Unlike the L(E) same mice, we found no reduction in L-selectin on circulating leukocytes and leukocyte subtypes in L(E) homo mice receiving DFA. These results indicate the L(E) homo mice are the more appropriate strain to evaluate the dependence of the efficacy of DFA on proteolytic cleavage of L-selectin. Ongoing studies will pursue this direction.**

IMPACT

Summary of Key Research Accomplishments

- 40mg/kg DFA as minimal effective dose required for L-selectin sheddase activity. This dose was used for all subsequent studies
- DFA does not exert adverse effects on animal health.
- Efficacy of DFA was demonstrated after either mild or severe SCI as evidenced by improved locomotor recovery.
- DFA, when given either immediately after SCI or at 3 hours post SCI improved locomotor recovery.However, no benefit was seen when treatment was initiated at 8 hours post –SCI.
- Multiple doses of DFA does not improve neurological outcomes and resulted in toxicity and increased mortality.

- DFA, delivered at 3 hours after a severe SCI, resulted in greater white matter sparing and reduced lesion volume .
- DFA did not alter the emergence of bladder dysfunction that accompanied SCI, based upon multiple measures including awake cystometry.

Conclusions
Using a mouse model of SCI and DFA at a dose of 40mg/kg,i.p., we conclude the following:

- This is the minimal effective dose to induce L-selectin shedding in the plasma and spinal cord following SCI.
- DFA improves locomotor recovery in both mild and severe SCI when administered within 3 hours post injury and has no overt adverse effects on animal health.
- DFA improves white matter sparing following severe SCI when administered within 3 hours post-injury
- DFA reduces the lesion volume following severe SCI when administered within 3 hours post-injury
- DFA does not alter the emergence of bladder dysfunction following severe SCI

Impact on the development of principal disciplines of the project:
Anti-inflammatory strategies for SCI have yet to be fully realized. In this study we demonstrate that abrogating L-selectin function early after SCI improves long-term recovery and tissue sparing. The reported results elucidate the role of L-selectin in inflammation and associated damage after SCI. Furthermore, the therapeutic window for DFA suggests a critical period in acute inflammation after SCI that contributes to long-term deficits.

Impact on other disciplines:
Targeting L-selectin function as a therapeutic strategy, via shedding with DFA, can likely be extended to other models of central nervous system trauma, such as traumatic brain injury, that are marked by extensive inflammation and immune cell infiltration. These data also contribute to the collective knowledge on the role of L-selectin in inflammation.

Impact on technology transfer
The collective results may guide the development of future non-steroidal anti-inflammatory drugs that induce L-selectin shedding.

Impact on society beyond science and technology
Nothing to Report

CHANGES/PROBLEMS
During the course of evaluating L-selectin shedding in L(E) same mice for Specific Aim 4, we discovered that this strain of mice was susceptible to modest shedding and could not be used to evaluate the efficacy of DFA and its dependence on proteolytic cleavage of L-selectin. To address this concern, we evaluated the parental strain, term L(E) homo, for the L(E) same mouse line. We identified absence of L-selectin shedding in response to DFA in the L(E) homo strain of mice. In order to generate sufficient mice to evaluate the L(E) homo mice in the context of SCI, new breeding cages were established. However, this resulted in a delay of approximately 4-6 months. Ongoing studies are now conducting studies, using the L(E) homo mice treated with and without DFA.

PRODUCTS

Journal publications. A manuscript including the reported data is under preparation and we anticipate submission in 1-2 months. Federal support will be acknowledged.

Conference presentations.

1. McCreedy DA, Sontag CJ, Lee SM, Martinez AF, Fandel TM, Trivedi A, Rosen SD, Noble-Haeusslein LJ. Targeting shedding of L-selectin on neutrophils as a treatment for spinal cord injury. Poster presentation. National Neurotrauma Society symposium. June 2016, Lexington, KY.

2. McCreedy DA, Sontag CJ, Lee SM, Martinez AF, Fandel TM, Rosen SD, Noble-Haeusslein LJ. The role of L-selectin in leukocyte recruitment and secondary pathogenesis following spinal cord injury. Poster presentation. Society for Neuroscience. October 2015, Chicago, IL.

3. Sontag, CJ, Lee SM, Rosen SD, Noble-Haeusslein LJ. Pharmacologically targeting L-selectin improves outcomes following spinal cord injury. Poster presentation. National Neurotrauma Society symposium. July 2014.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name:	Linda Noble-Haeusslein and Steven Rosen
Project Role:	PI and CoPI, respectively
Researcher Identifier	
Nearest person month worked:	
Contribution to Project:	48
Funding Support:	

Name:	Chris Sontag
Project Role:	Post-doctoral fellow
Researcher Identifier	
Nearest person month worked:	18
Contribution to Project:	Dr. Sontag performed the experiments and analyses in Specific Aims 1 and 2 to evaluate the dosage, efficacy, and therapeutic window of DFA.
Funding Support:	

Name:	Dylan McCreedy
Project Role:	Post-doctoral fellow
Researcher Identifier	
Nearest person month worked:	18
Contribution to Project:	Dr. McCreedy developed the flow cytometry methods and analyses used for Specific Aims 1b and 4.
Funding Support:	Recipient of a National Institutes of Neurological Diseases and Disorders post-doctoral fellowship in 2016

Name:	Aida Martinez
Project Role:	Staff Research Assistant
Researcher Identifier	
Nearest person month worked:	1
Contribution to Project:	Ms. Martinez performed the morphometric analyses in Specific Aim 1d.
Funding Support:	

Name:	Alpa Mahuvakar
Project Role:	Senior Research Scientist
Researcher Identifier	
Nearest person month worked:	1
Contribution to Project:	Dr. Trivedi helped establish the behavioral analyses used to determine neurologic recovery in spinal cord injured mice.
Funding Support:	

Name:	Thomas Fandel
Project Role:	Research Specialist
Researcher Identifier	
Nearest person month worked:	3

Contribution to Project:	Dr. Fandel performed the cystometry experiments and analyses to evaluate bladder function in Specific Aims 1c and 3
Funding Support:	

Change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period

New funding

Medical Rehabilitation Research Resource Boninger/Rando (Co-PIs) 09/01/15 - 8/31/2020
Alliance for Regenerative Rehabilitation Research & Training (AR3T)
The overarching goal of the Alliance for Regenerative Rehabilitation Research & Training (AR3T) is to expand scientific knowledge, expertise and methodologies across the domains of rehabilitation science and regenerative medicine, thus improving the lives of individuals with disabilities.
Role: PI of subcontract

Craig H Neilsen Foundation Chang (PI) 01/01/14 - 12/31/2016
The roles of MMPs and therapeutic intervention in spinal cord injury
The goal of this project is to identify and define the roles of MMPs in the acute and repair phases of TBI and to evaluate selective inhibitors in a mouse model of SCI. These studies rely on novel affinity resin that binds only the active forms of MMPs, which combined with proteomics identifies and quantifies active MMPs. This methodology, combined with new selective MMP inhibitors, will define mediators of early secondary injury and target those that are involved in wound healing.
Role: PI of Subcontract

R01NS077767 Noble (PI) 07/01/2012 - 06/30/2017
NIH/NINDS
Determinants of vulnerability and recovery after trauma to the developing brain
Using genetic and pharmacologic approaches in a murine model of TBI, the goal of this study is determine how unchecked, neutrophil elastase (NE)-directed proteolysis establishes an environment that is unfavorable to recovery and if early blockade of this activity supports cognitive recovery.
Role: PI

Private Gift/UCSF Noble/Ferriero (Co-PIs) 01/01/2013 - 12/30/2016
Exercise as a strategy to improve cognitive outcome in a murine model of repeated concussive insults
The goals of this study are to develop a murine, adolescent model of concussion injury, focusing on both structural and functional characterization, and to determine if defined voluntary exercise, rescues behavioral deficits.
Role: Co-PI

- **.Completed studies**

RC1 NS068200 (Co-PIs, Kriegstein/Noble) 10/01/2009 – 09/30/2012
NINDS
Spinal Cord Injury: Targeting Local Inhibition To Improve Outcome
Performance Period:
To determine whether MGE progenitor cells, grafted below the level of a spinal cord injury, can provide inhibitory modulation of spinal circuits and provide inhibitory modulation of spinal circuits reducing neurogenic bladder dysfunction.

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Other organizations were involved as partners
Nothing to Report

APPENDICES
All data, reported below, reflect the mean + SEM.

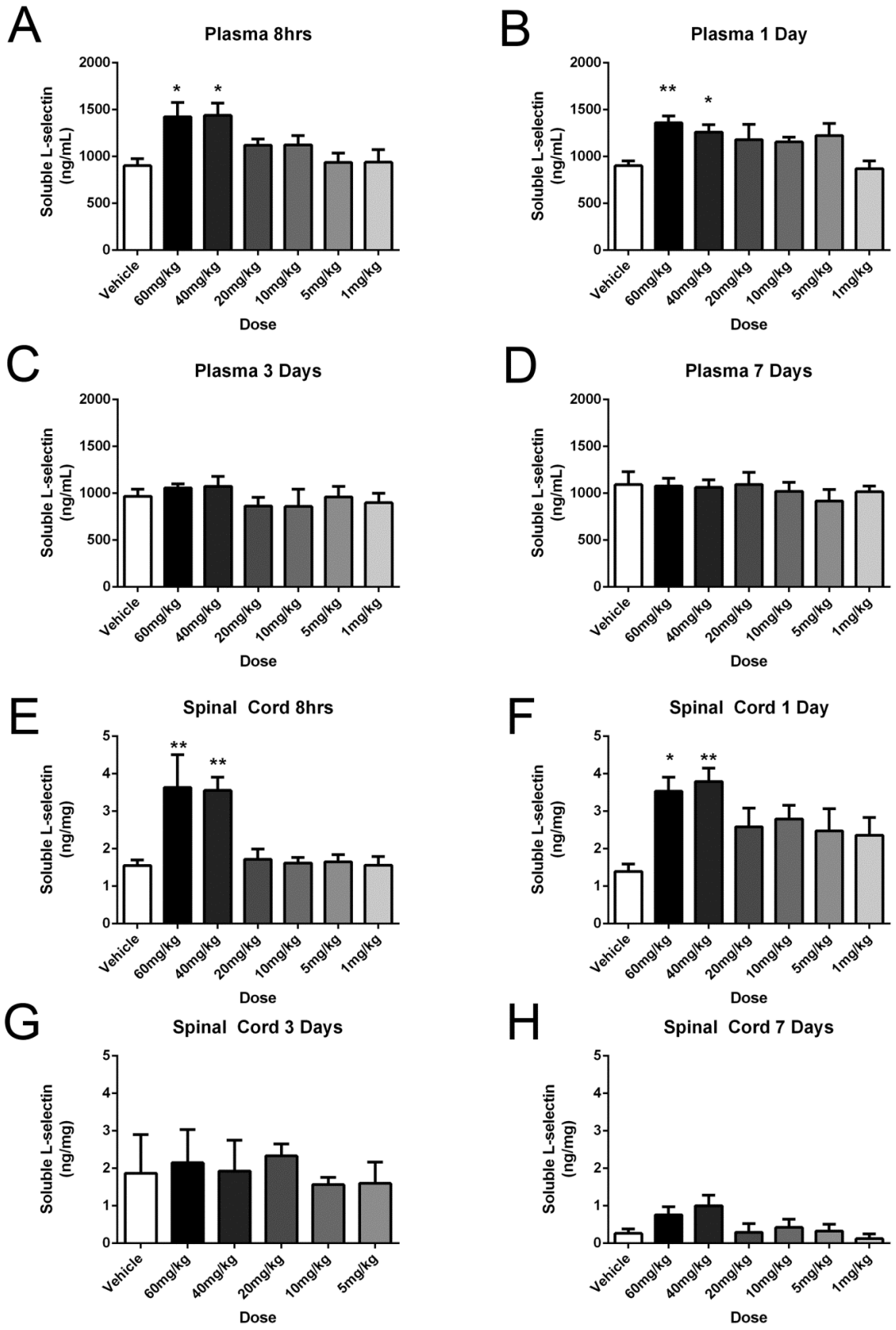

Figure 1: 60mg/kg and 40mg/kg DFA induce shedding of L-selectin from leukocytes in plasma and spinal cord following spinal cord injury as detected by ELISA. N=5/group. One-way ANOVA followed by Dunnett's post-hoc test. *p<0.05, **p<0.01.

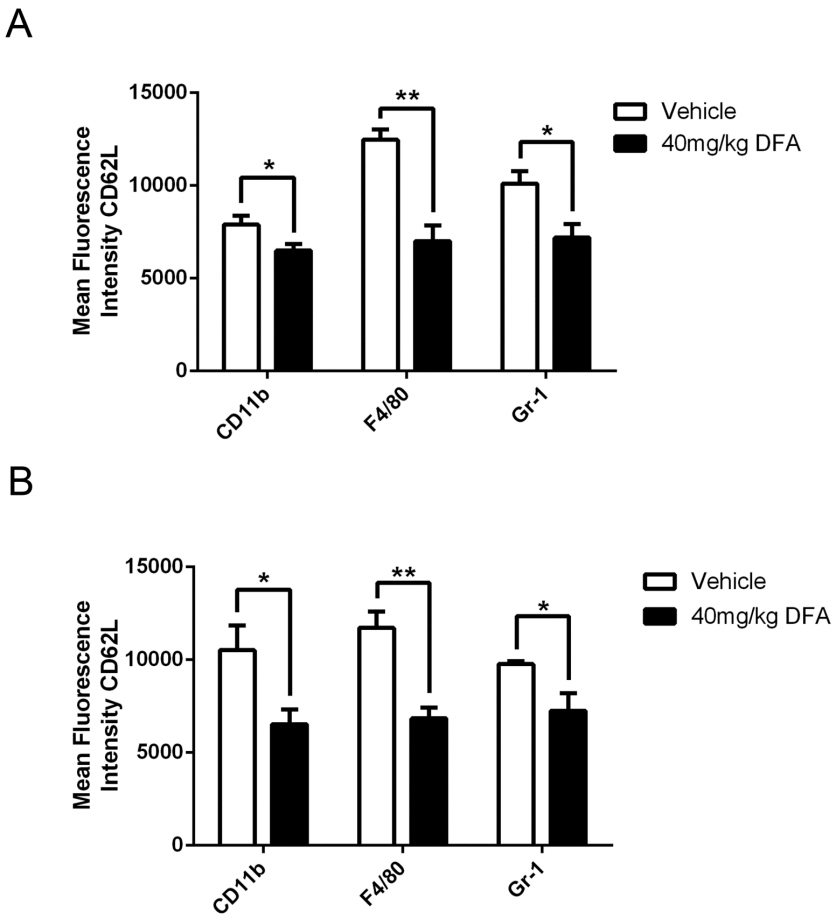

Figure 2: Flow cytometry with broad-labelling markers confirms shedding of L-selectin from leukocytes in the plasma and spinal cord by 40mg/kg DFA following mild spinal cord injury. N=5/group. One-way ANOVA followed by Dunnett's post-hoc test. *p<0.05, **p<0.01.

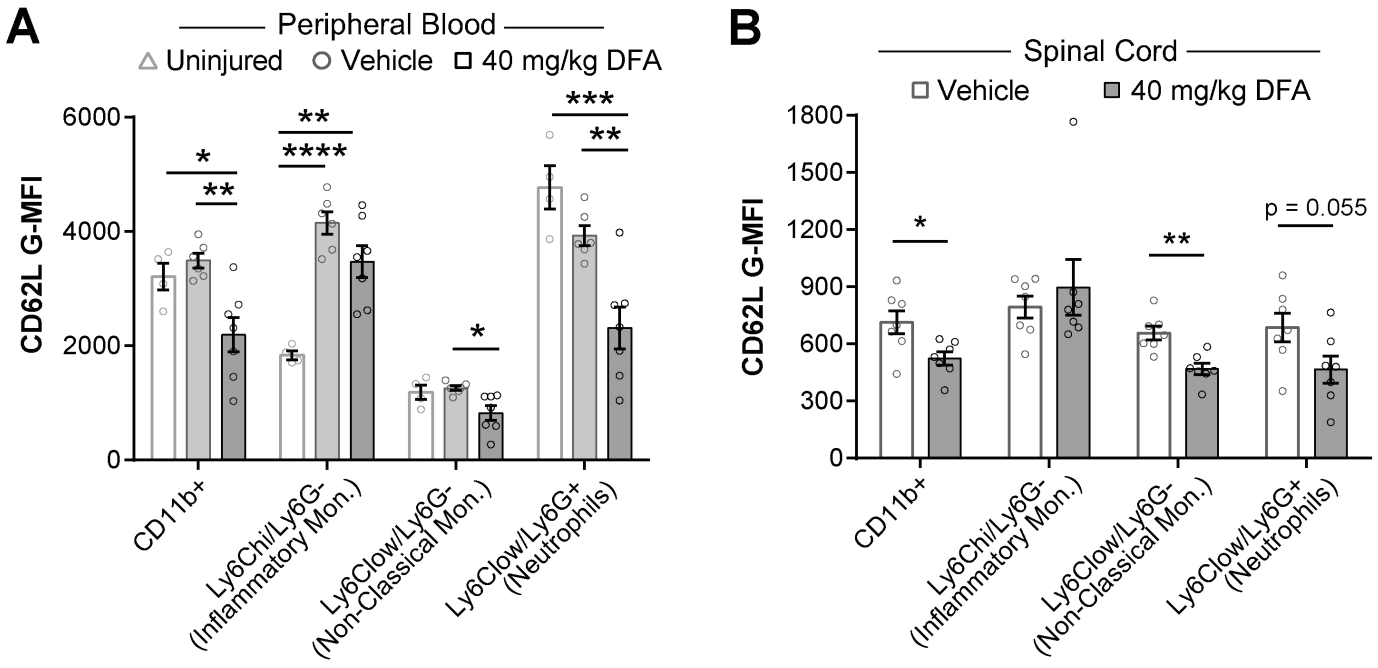

Figure 3: Flow cytometry using subtype specific markers demonstrates shedding of L-selectin from non-classical monocytes and neutrophils in the plasma and spinal cord by 40mg/kg DFA following severe spinal cord injury. N=7/SCI/group and 4/uninjured/group. Unpaired two-way Student's T-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

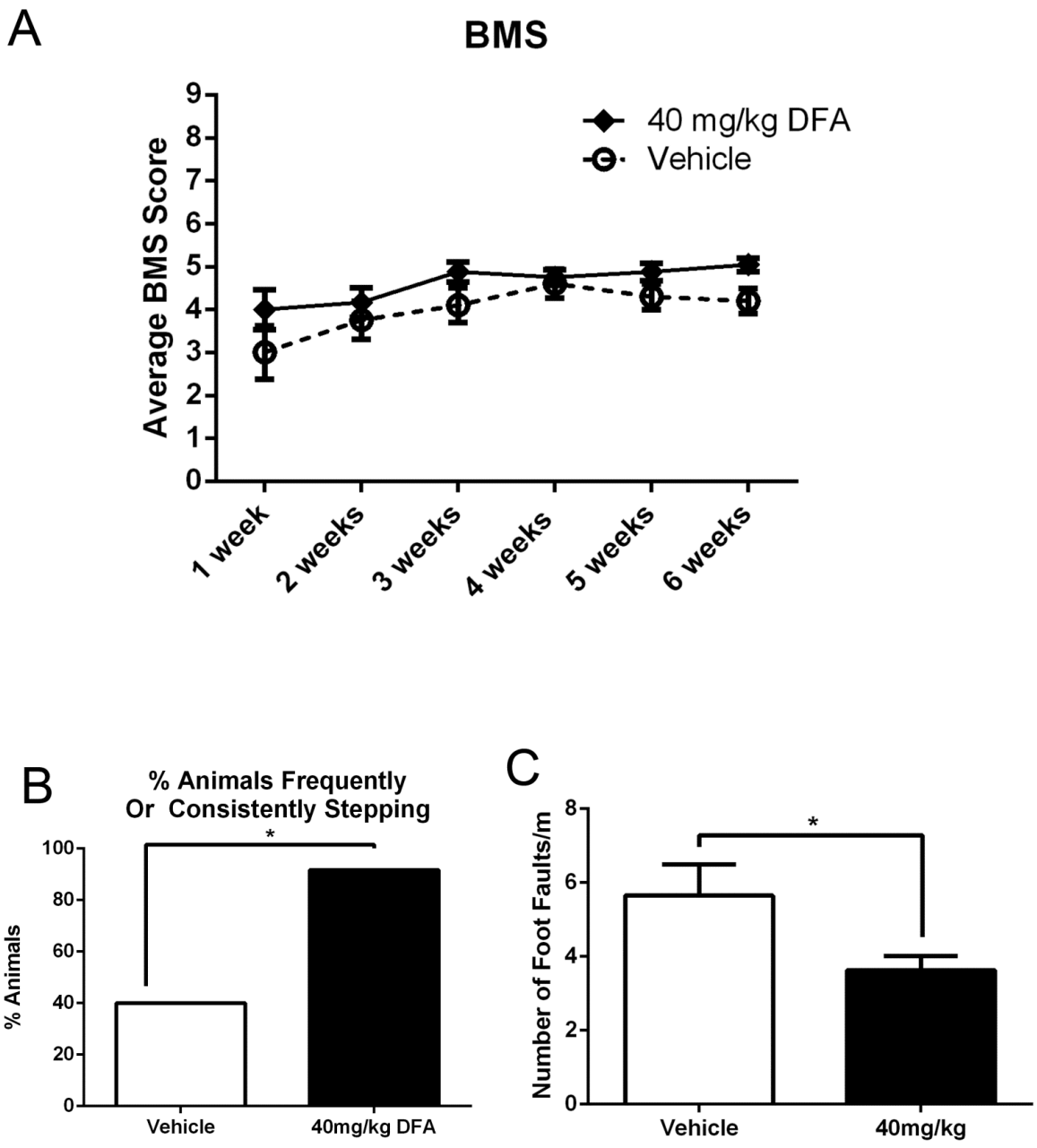

Figure 4: 40mg/kg DFA administered immediately post-mild SCI improves locomotor recovery. A) Average BMS scores. N=15/group. Two-way repeated measures ANOVA. No significant effect of treatment was observed ($p>0.05$). B) Percentage of animals frequently or consistently stepping in each group. N=15/group. Chi square frequency analysis with Fischer's exact test. C) Grid walk test analysis. N=9 for vehicle and 12 for DFA. Kruskal-Wallis test. *$p<0.05$.

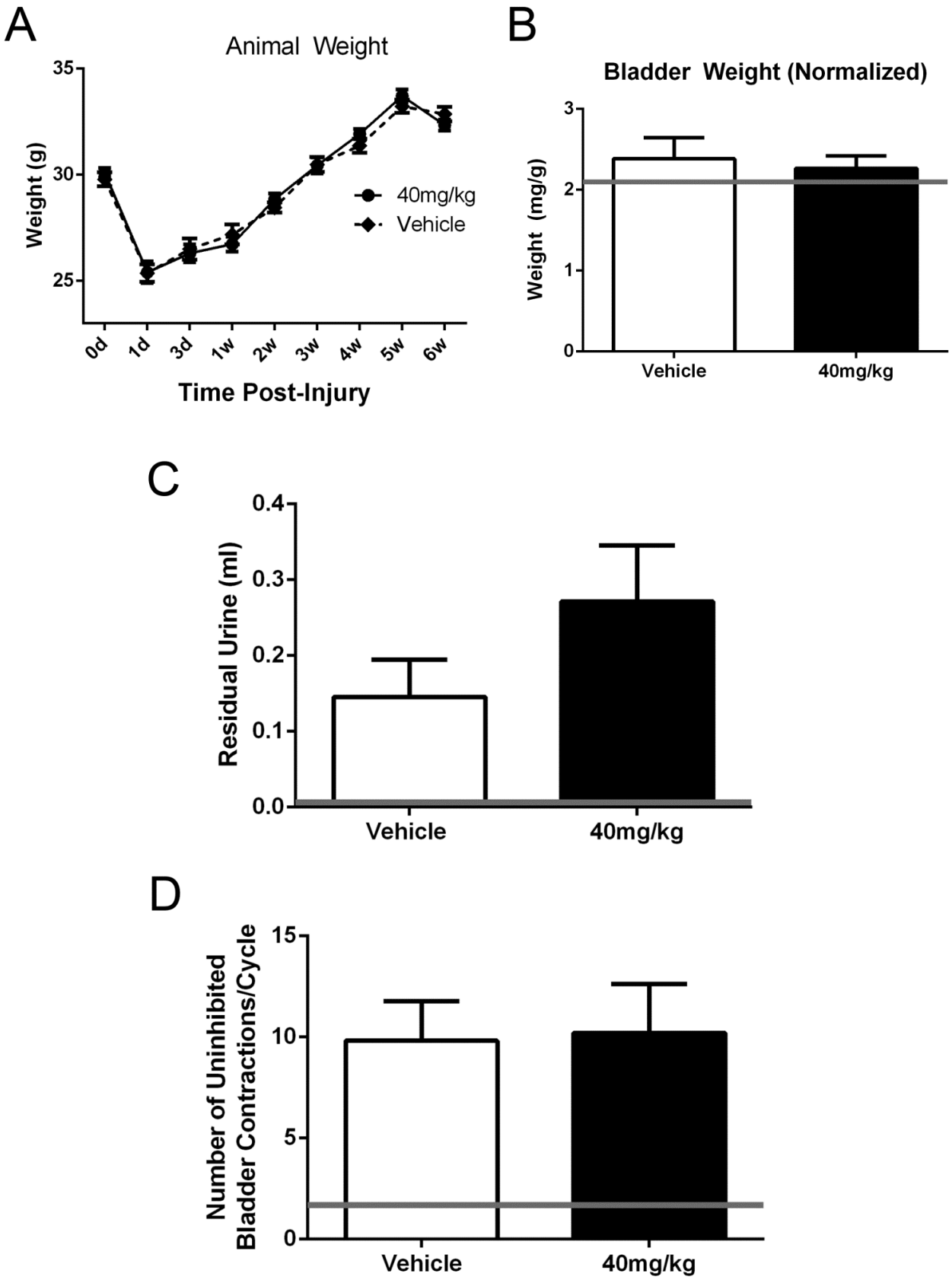

Figure 5: 40mg/kg DFA administration post-mild SCI does not influence animal weight, bladder weight, or measures of bladder function. A) Animal weight post-SCI. N=15/group. Two-way repeated measures ANOVA. No significant effect of treatment was observed ($p>0.05$). B) Bladder weights at 6 weeks post-SCI. N=9/group. One-tailed Student's T-test. C-D) Awake cystometry analysis. N=9/group. Kruskal-Wallis test. *$p<0.05$.

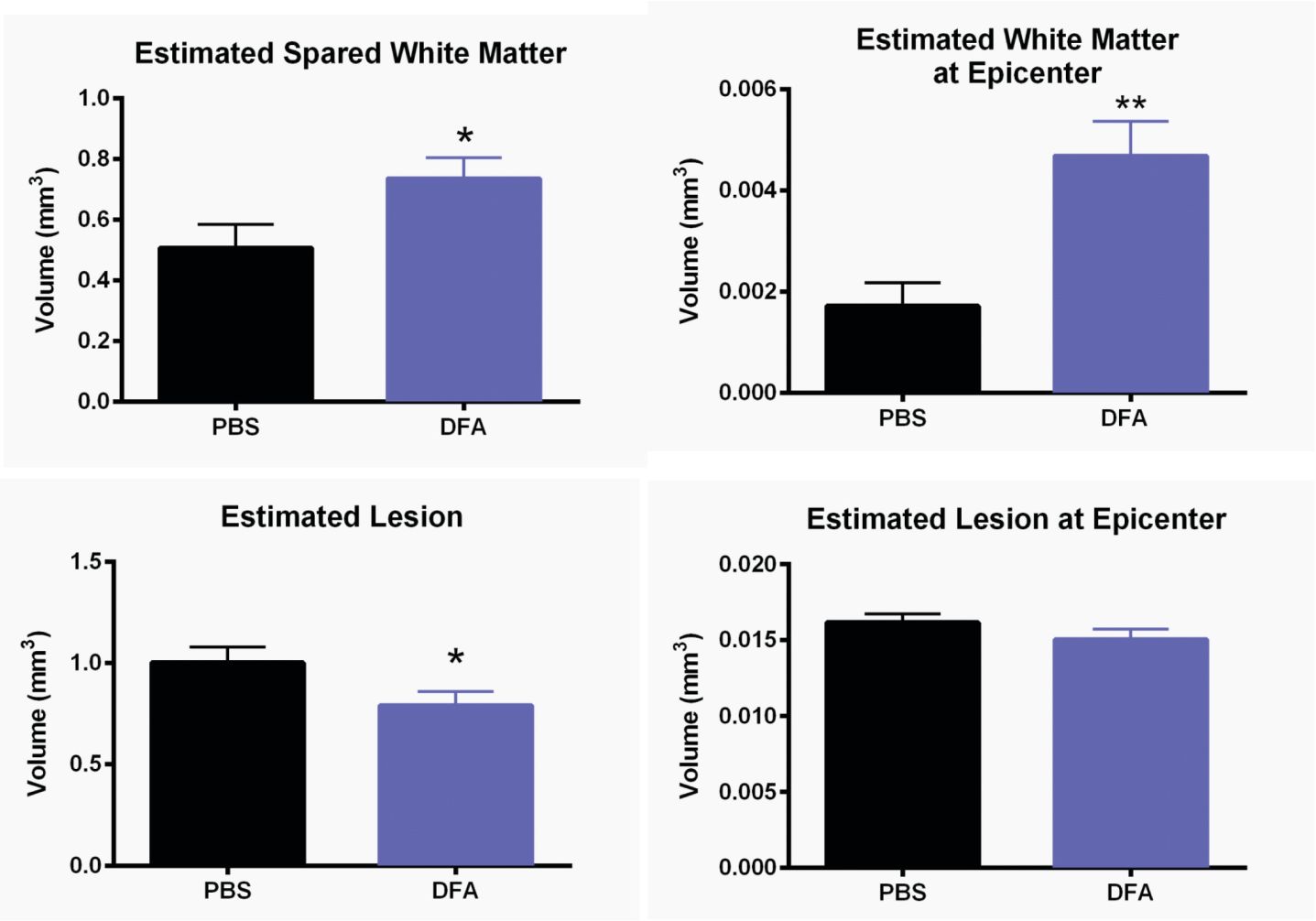

Figure 6: 40 mg/kg DFA administration 3 hours post-severe SCI enhances white matter sparing and reduces the lesion volume. N=14 for DFA and 7 for vehicle. Mann-Whitney test. *p<0.05, **p<0.01.

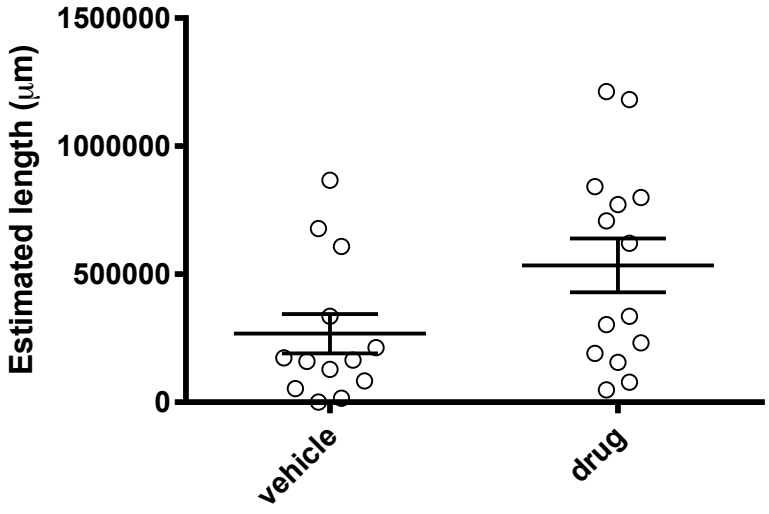

Figure 7: Estimated length of serotonergic (5-HT) fibers with 40 mg/kg DFA administration at 3 hours post-severe SCI. N=13-14/group. Mann-Whitney test. p=0.054.

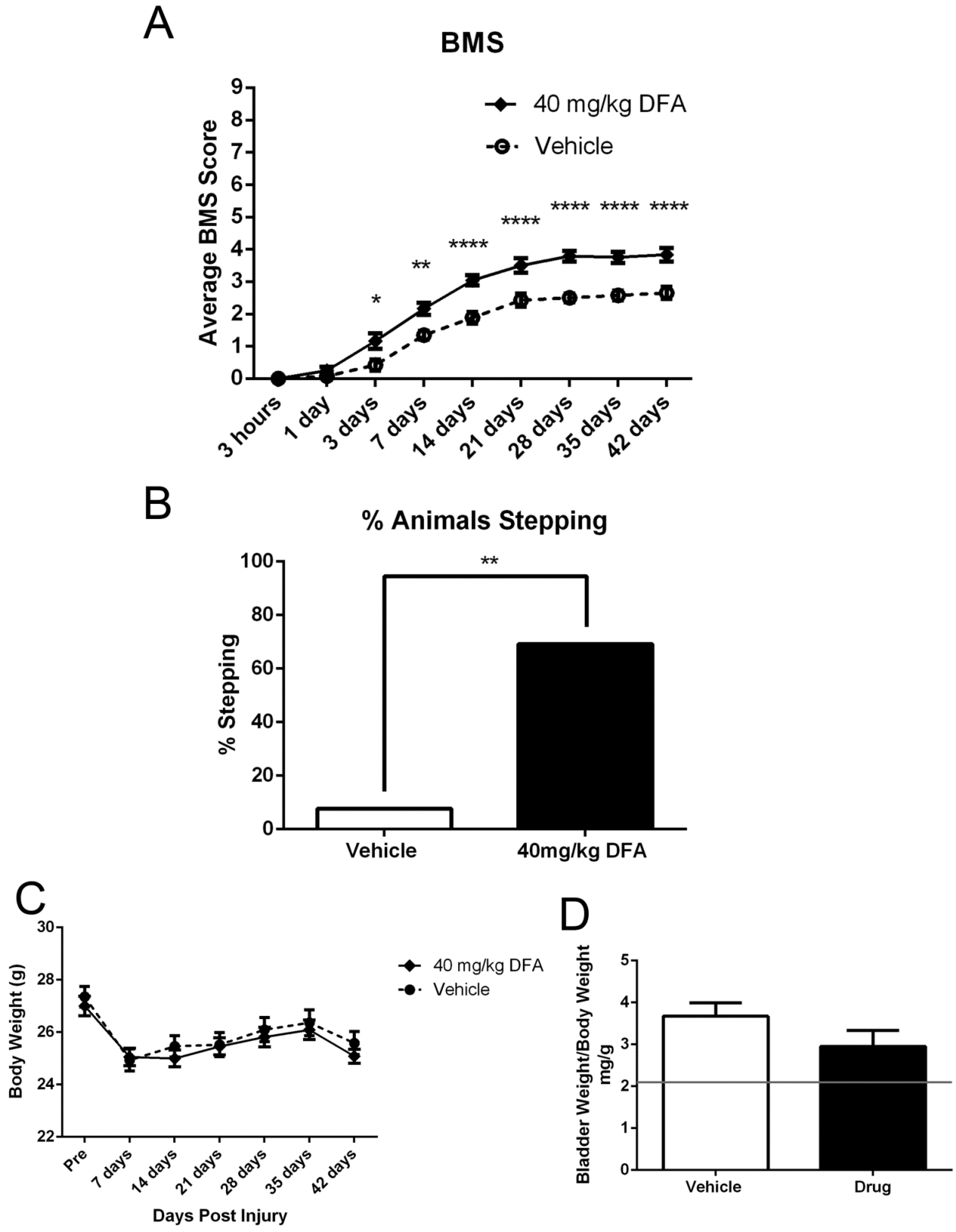

Figure 8: 40mg/kg DFA administration immediately post-severe SCI improves locomotor recovery and does not influence animal weight or bladder weight. N=15/group. A) Average BMS scores. Two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. B) Percentage of animals stepping in each group. Chi square frequency analysis with Fischer's exact test. C) Animal weight post-SCI. Two-way repeated measures ANOVA. No significant effect of treatment was observed ($p>0.05$). D) Bladder weight at 6 weeks post-SCI. One-tailed Student's T-test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

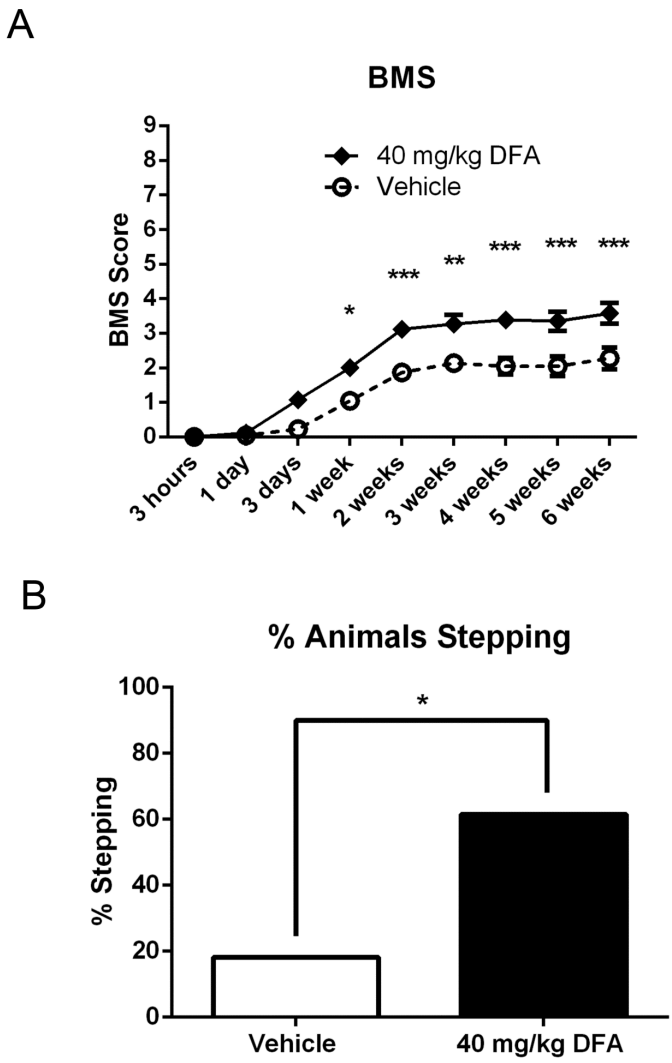

Figure 9: 40mg/kg DFA administration 3 hours post-severe SCI improves locomotor recovery. N=15/group. A) Average BMS scores. Two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. B) Percentage of animals stepping in each group. Chi square frequency analysis with Fischer's exact test. *p<0.05, **p<0.01, ***p<0.001.

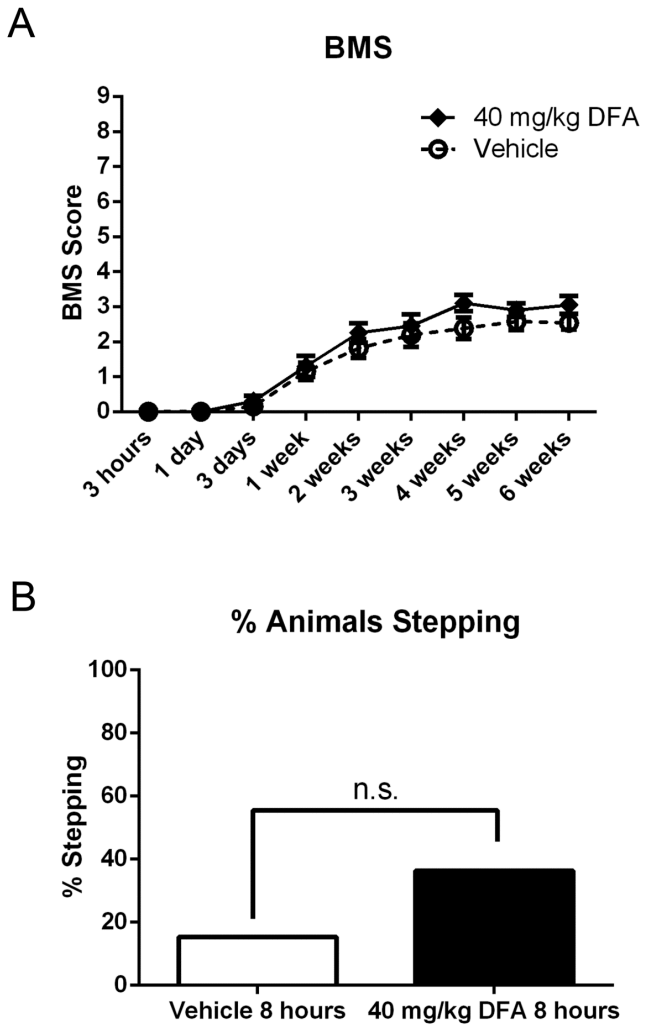

Figure 10: 40mg/kg DFA administration 8 hours post-severe SCI does not improve locomotor recovery. N=15/group. A) Average BMS scores. Two-way repeated measures ANOVA. No significant effect of treatment was observed ($p>0.05$). B) Percentage of animals stepping in each group. Chi square frequency analysis with Fischer's exact test. n.s. = not significant.

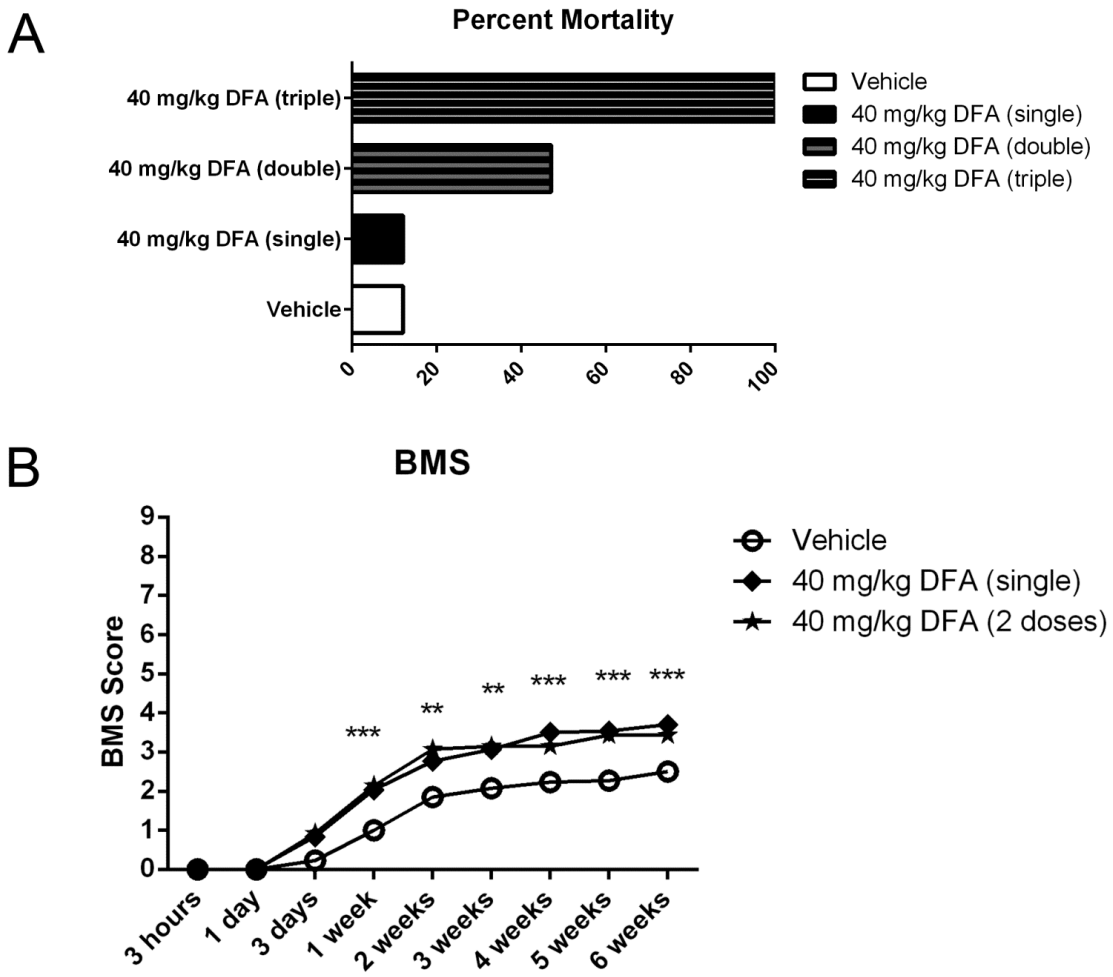

Figure 11: Multiple doses of 40mg/kg DFA administered beginning 3 hours post-severe SCI are toxic and do not confer benefits to locomotor recovery over a single dose. A) Percent mortality for single, double, and triple dose regimens. N=15/group. B) Average BMS scores. N=13 for vehicle, 15 for single dose, and 7 for two doses. Two-way repeated measures ANOVA followed by Sidak's multiple comparisons test. *p<0.05, **p<0.01, ***p<0.001.

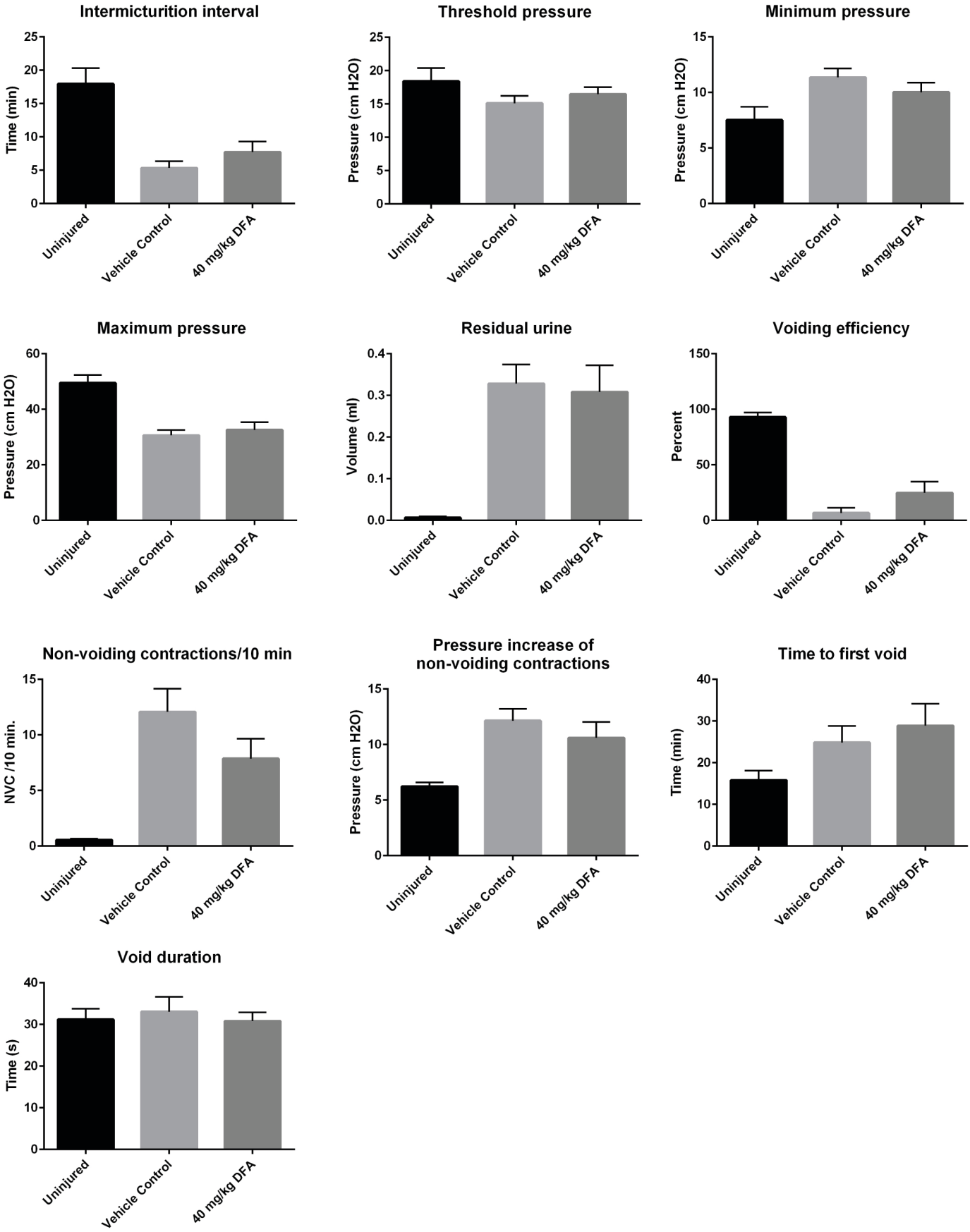

Figure 12: 40 mg/kg DFA administration 3 hours post-severe SCI does not alter urologic function compared to vehicle controls. N=13-15/group. Unpaired Student's T-tests between vehicle and DFA treated groups. *p<0.05.

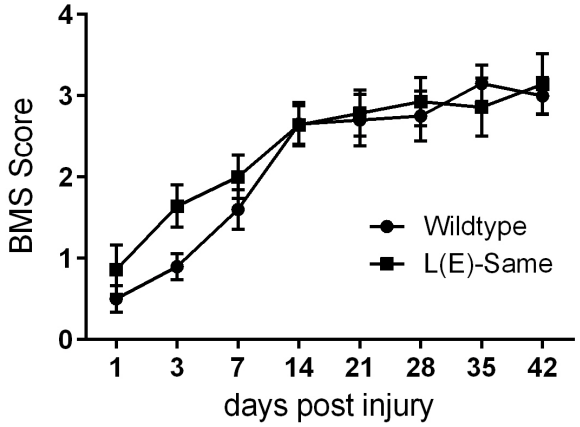

Figure 13: Similar neurological recovery in L(E) same mice versus wild-type mice following moderate-severe spinal cord injury. N=7-10/group. Two-way repeated measures ANOVA. No significant effect of treatment was observed ($p>0.05$).

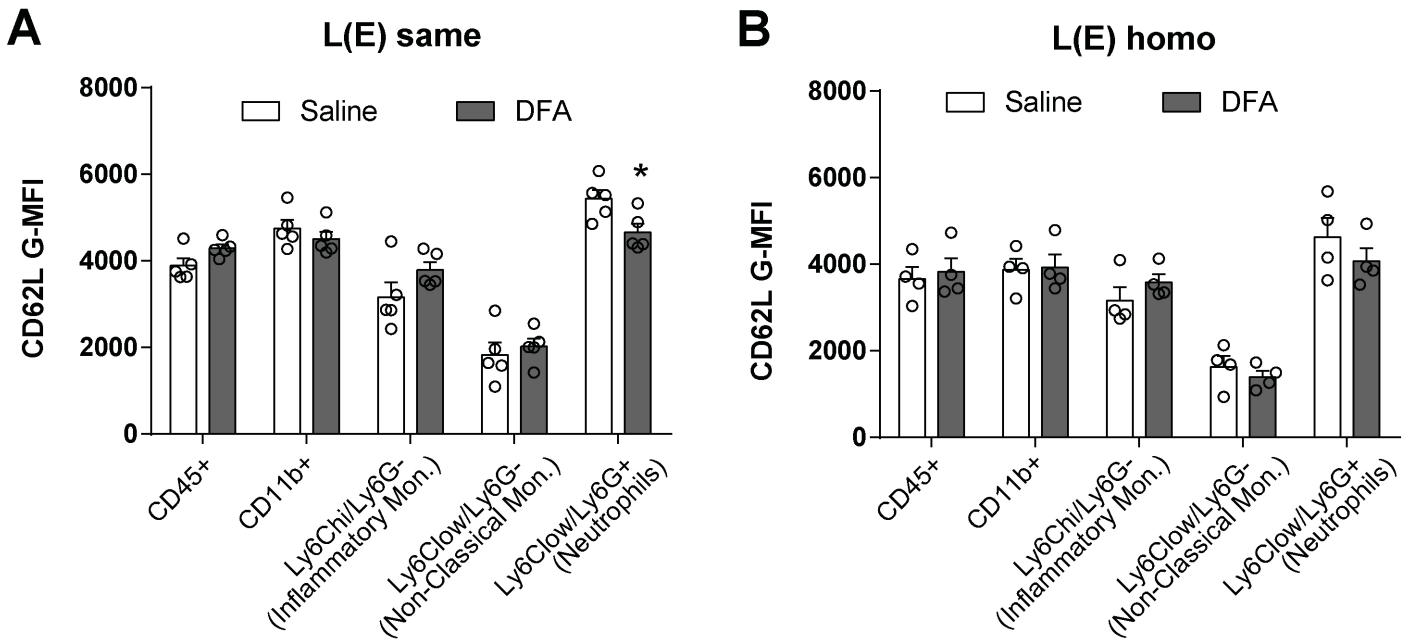

Figure 14: A) Flow cytometry analysis reveals significant loss of L-selectin on neutrophils from L(E) same mice at 8 hours post-administration of 40 mg/kg DFA. N=5/group. Unpaired two-way Student's T-test. B) No loss of L-selectin is observed on circulating leukocytes from L(E) homo mice at 8 hours post-administration of 40 mg/kg DFA. N=4/group. Unpaired two-way Student's T-test. *$p<0.05$.